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Factor VIIa Inducing Expression of Cyr61

Tissue factor (TF) is a cellular receptor for the plasma clotting factor VII(a), and the TF-VII(a) complex initiates the coagulation protease cascade (1). Recent reports from a number of laboratories indicate that TF influences an array of important biological functions other than coagulation, such as angiogenesis (2,3), embryo vascularization (4) and tumor metastasis (2,5,6). At present, it is unclear how TF contributes to these biological processes. The extracellular domain of TF consists of two fibronectin type III like modules (7), as in the typical class II cytokine receptor extracellular domain, raising the possibility that TF may play a role in signal transduction, the primary function of cytokine receptor. However, TF has a very short cytoplasmic domain (only 21 amino acid residues in length) and lacks membrane-proximal motifs that mediate binding of the non-receptor Janus kinases (Jaks) that are essential for cytokine receptor signalling (8). Nonetheless, several biochemical findings suggest a signal transduction function for TF. Analysis of the human TF protein sequence revealed a putative phosphorylation site in the cytoplasmic domain which is conserved in mouse, rat and rabbit TF (9). Specific serine residues in the cytoplasmic tail of TF are phosphorylated in cells following stimulation with protein kinase C activator (9). Mody and Carson (10) reported that the human TF cytoplasmic tail is phosphorylated *in vitro* at multiple sites when incubated with lysates of U87-MG cells. A potential role for the TF cytoplasmic domain in signal transduction is also indicated in studies that showed prometastatic function of TF is critically dependent on the TF cytoplasmic domain (5,6). Further, TF cytoplasmic domain is shown to interact with actin-binding protein 280 (ABP-280) and supports cell adhesion and migration through recruitment of ABP-280 to TF-mediated adhesion contacts (11).

However, a growing number of recent reports provide evidence that TF also participates in cell signalling by serving as a cofactor for its physiological ligand VIIa in an extracellular signalling by proteolytic mechanism. For example, binding of VIIa to cell surface TF is shown to induce intracellular Ca^{2+} oscillations in a number of TF expressing cells (12,13), transient phosphorylation of tyrosine in monocytes (14), activation of MAP kinase (15,16), alteration in gene expression in fibroblasts (17,18) and enhanced expression of urokinase receptor in tumor cells (19). Since catalytically inactive VIIa (VIIai) fails to induce many of the above signalling responses, from Ca^{2+} oscillations (13) to MAP kinase activation (15) and gene induction (15), it

appears that the catalytic activity of VIIa is required for TF-VIIa-mediated signal transduction. Further, recent studies of Sorensen et al. (16) show that the cytoplasmic TF domain with its putative sites for regulatory modifications is not required for VIIa-induced MAP kinase activation. At present, not much is known about signalling pathway(s) that are induced by proteolytically active VIIa and how the signals generated by VIIa could contribute to angiogenesis and tumor metastasis.

To study temporal program of transcription that underlies the VIIa-induced response, in the present study, we have examined the response of human fibroblasts to VIIa using a cDNA microarray. The data revealed that the cellular expression of several genes was detectably altered in fibroblasts upon exposure of to VIIa. One such gene is *Cyr61*, a growth factor-inducible immediate early gene, whose product is shown to promote cell adhesion, augment growth factor-induced DNA synthesis and stimulate cell migration in fibroblasts and endothelial cells (20).

MATERIALS AND METHODS

Cell Culture. A fibroblast cell line, WI-38 (obtained from ATCC, Rockville, MD), was grown in Dulbecco's modified Eagle medium (GLUTAMAX with high glucose from GIBCO BRL Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Cellgro, xxx), 1% penicillin and streptomycin (Bio Whittaker, xxx), and 1% L-glutamine (Bio Whittaker). When the cells reached about 80% confluency, the serum containing growth medium was removed and the cells were washed once with DMEM and the incubated with DMEM for 20 to 24 hours to make the cells quiescent.

Proteins. Recombinant human VIIa, a gift from Novo Nordisk (Gentofte, Denmark), was reconstituted in sterile water at a concentration of 1 to 1.3 mg/ml. The stock VIIa solutions were checked for contaminating trace levels of endotoxin using limulus amebocyte lysate (Bio Whittaker) and none was detected (detection level 30 pg). Recombinant tick anticoagulant protein (TAP) was kindly provided by George Vlasuk (Corvas, San Diego, CA) and recombinant hirudin was obtained from either Sigma (St.Louis, MO) or Calbiochem (San Diego, CA). Purified human factor Xa and thrombin were obtained from Enzyme Research Laboratories (Southbend, IN).

cDNA microarray. WI-38 cells were cultured to 80% confluency and serum deprived for 24 hours to enter quiescent state as described above. The culture medium was replaced with fresh serum-free DMEM (supplemented with 5 mM $CaCl_2$) and allowed to stabilize for 2 h in culture incubator. Then, the cells were treated with purified recombinant VIIa (5 μg/ml) for 90 min. At the end of 90 min treatment, total RNA was isolated from untreated (control) and VIIa-treated cells using Trizol (GIBCO BRL). Poly (A) RNA was purified by a double pass over Oligo Tex mRNA isolation columns as described in manufacturer's technical bulletin (Qiagen). Eight hundred ng (800 ng) of highly purified poly (A) RNA from the control and VIIa-treated cells were sent for cDNA microarray analysis service (Human UniGEM V microarray, Genome Systems Inc, St. Louis, MO).

Northern Blot Analysis. Total RNA was prepared using TRIZOL reagent from quiescent monolayers of WI-38 cells that were exposed to VIIa and other materials as described in Results. Northern blot analysis was

carried out using standard procedure. Briefly, 10 µg of total RNA was size fractionated by gel electrophoresis in 1% agarose/6% formaldehyde gels and transferred onto the nitrocellulose membrane by a capillary blot method. Northern blots were prehybridized at 42°C with a solution containing 50% formamide, 5 x SSC, 50 mM Tris.HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 1% polyvinylpyrrolidone, 1% Ficoll, 25 mM EDTA, 100 µg/ml denatured salmon sperm DNA and 1% BSA and hybridized with ^{32}P-labeled Cyr61 cDNA probe (10^6 cpm/ml). The hybridized membranes were exposed to either Dupont NEF or Fuji RX X-ray film. For quantification purposes, the membranes were exposed to phosphor screen for 1 to 4 h, and the exposed screens were analyzed in a PhosphorImger (Molecular Dynamics) using "Image-quant" software. To obtain mean values, the units (counts) obtained from different experiments were normalized to an internal control (counts present in control-treated sample).

Chromogenic Assay. WI-38 cells were cultured in 96-well culture plate and made them quiescent as described above. After washing the cells, FVIIa (5 µg/ml) in 100 µl of calcium containing buffer was added to the culture wells containing cells or wells coated with buffer (no cells). After 30 min incubation, 25 µl of chromogenic substrates for factor Xa and thrombin, i.e., Chromozym X and Chromozym TH were added to the wells. After 3 h of color development, the plate was read in a microplate reader. As controls, cells were incubated with trace concentrations of factor Xa (50 to 0.1 ng/ml) or thrombin (0.1 to 0.002 U/ml). No differences were found in absorbance at 450 nm between VIIa added to cells or VIIa added to wells not containing cells. The reading was lower than the readings obtained with lowest concentration of factor Xa or thrombin and represents VIIa chromogenic activity.

RESULTS

cDNA microarray. Quiescent fibroblasts were exposed to a control serum-free medium or the serum-free medium supplemented with VIIa (5 μg/ml) for 90 min (three T-75 flasks for each treatment). After the treatment, total RNA was harvested and poly (A) RNA was isolated. Six hundred ng of mRNA was labeled with either Cy3 or Cy5 fluorescence and then hybridized to the UniGem Human V chip containing 8,000 sequence verified ESTs, representing up to 5,000 known human genes (service performed by Genome System Inc for a fee). The control plate, in which known concentrations of reference cDNA was spiked into the probe generation reaction to measure sensitivity and monitor the reverse transcription reaction, purification, determine hybridization efficiency and overall view of the quality and performance of the assay indicated the success of hybridization process. Global analysis of experimental data revealed minimal differences in hybridization signals between the control and VIIa-treated samples. Only a small number of genes showed moderate differential expression. We found upregulation of 5 genes (3.5 to 2-fold higher in VIIa treatment) whereas one gene was down-regulated upon VIIa treatment (2.4-fold lower) (+/- 2 is a conservative estimate for determining the minimum magnitude of real ratios). The identity of the 3.5-fold upregulated gene was not revealed due to the proprietary nature. Other VIIa- upregulated genes are *Cyr61* (2.5-fold), dopamine D2 receptor (2.2-fold), *EST Incyte PD 395116* (2-fold) and P2U nucleotide receptor (2-fold). It is interesting to note that *CTGF*, a gene belonging to the *Cyr61* family, was 1.8-fold higher in VIIa-treated cells compared to control cells. The down-regulated transcript in VIIa-treated cells was *EST PD674714*. Since, at present, the identity of the 3.5-fold upregulated transcript is not known, we selected *Cyr61*, whose differential expression ratio is higher than other possible upregulated genes, for further analysis.

Confirmation of differential expression of *Cyr61*. To validate the data obtained in microarray, we have subjected the RNA samples from the control and VIIa-treated cells (the same RNA samples that have been used to prepare poly (A) RNA for probe generation in the microarray) to Northern blot analysis and probed with radiolabeled *Cyr61* cDNA. The data show that *Cyr61* cDNA probe hybridized to a single transcript (approximately 2.0 kb) of RNA isolated from the control and VIIa-treated cells. However, the intensity of

hybridization signal was much higher in RNA isolated from VIIa-treated cells (Fig. 1). Quantitation of hybridization signal revealed that expression of *Cyr61* was 2.8-fold higher in cells exposed to VIIa over the control treated cells.

Kinetics of VIIa-induced expression of *Cyr61*. To determine the kinetics of *Cyr61* expression, quiescent fibroblasts were treated for varying time periods with 5 μg/ml VIIa. Total RNA was extracted and subjected to Northern blot analysis. As shown in Fig. 2, *Cyr61* expression was increased in time-dependent manner in VIIa-treated cells. The expression was peaked at about 45 min and thereafter declined to the base level in 2 to 3 h. Since it had been reported that expression of *Cyr61* in mouse fibroblasts after stimulation with serum and growth factor was sustained for several hours (up to 8 to 10 h) before repression occurs (21), we have examined the effect of serum and PDGF on kinetics of *Cyr61* expression in quiescent human fibroblasts, WI-38. As shown in Fig. 2B, *Cyr61* is expressed only transiently upon stimulation with PDGF and become fully repressed 2 h after the addition of stimuli. Similar results obtained with serum-induced expression of *Cyr61* (data not shown).

Factor VIIa-dose dependent induced expression of *Cyr61*. To determine dose-dependency of VIIa, quiescent fibroblasts were treated with varying doses VIIa (0.1 to 5 μg/ml) for 45 min and then total RNA samples from the cells were subjected to Northern blot analysis. As shown in Fig. 3, treatment of fibroblasts with as low as 0.1 μg/ml VIIa was sufficient to induce the expression of *Cyr61* and a plasma concentration of VII(a) (0.5 μg/ml, 10 nM) resulted in a prominent response, close to the maximal.

Factor VIIa-catalytic activity is required for *Cyr61* induction. To test whether VIIa catalytic activity is required for the induction of *Cyr61*, WI-38 cells were treated with VIIa and active-site inactivated VIIa (VIIai) for 45 min and the expression of *Cyr61* was evaluated by Northern blot analysis. As shown in Fig. 4, VIIai failed to induce the expression of *Cyr61* suggesting the requirement of VIIa proteolytic activity. In this context, it may be important to point out that VIIai was shown to bind cell surface TF with the same or higher affinity than VIIa (22). It is unlikely that VIIa-induced expression of *Cyr61* in our experiments was the result of generation of down-stream coagulation factors, FXa and thrombin. By using sensitive chromogenic assays, we

found no evidence for the generation of factor Xa and thrombin in our experimental system (detection sensitivity 10 pg). Further, the specific inhibitors of factor Xa and thrombin, i.e., tick anticoagulant protein and hirudin, respectively, failed to abolish VIIa-induced expression of *Cyr61* (Fig. 5).

Involvement of transcriptional mechanism for the induction of *Cyr61* mRNA steady-state levels by VIIa. To investigate whether transcription is involved in VIIa-mediated increase in *Cyr61* mRNA steady-state levels, quiescent WI-38 cells were incubated with actinomycin-D (10 μg/ml) for 30 min before the addition of VIIa for 45 min. As shown in Fig.6, actinomycin-D inhibited the stimulator effect of VIIa. This finding indicates a transcriptional mechanism for induction of *Cyr61*.

To investigate whether *de novo* protein synthesis is required for the induction of *Cyr61* mRNA by VIIa, WI-38 cells were pretreated with the protein synthesis inhibitor cycloheximide before the cells were exposed to VIIa for 45 min. As shown in Fig. 6, the stimulatory effect of VIIa was not blocked by cycloheximide. In fact, cycloheximide markedly increased the VIIa-induced *Cyr61* mRNA steady-state levels.

DISCUSSION

The primary function of plasma clotting factor VIIa, upon its binding to TF on the cell surface, is to initiate blood coagulation. A number of recent studies suggest that VIIa binding to TF not only triggers the coagulation cascade but also leads to other cellular processes such as angiogenesis and tumor metastasis (2). It is not clear how VIIa-TF influences the complex biological processes. In case of tumor cell metastasis, it appears that the extracellular functions of the catalytically active VIIa-TF complex cooperate with specific functions of the TF cytoplasmic domain (6). Binding of VIIa to TF is shown to induce a number of intracellular signals (see (23)) Since catalytically inactive VIIa (VIIai) fails to induce many of the signalling responses, from Ca^{2+} oscillations (13) to MAP kinase activation (15) and gene induction (15), it appears that the catalytic activity of VIIa is required for TF-VIIa-mediated signal transduction. Further, the specific inhibitors for factor Xa and thrombin failed to inhibit the VIIa-induced signal transduction (15,16). Therefore, VIIa-induced signal transduction arise directly from VIIa proteolytic activity and not from possible generation of downstream activated coagulation factors, such as factor Xa and thrombin. At present, not much is known about signalling pathway(s) that are induced by proteolytically active VIIa and how the signals generated by VIIa could contribute to angiogenesis and tumor metastasis. One possibility is that VIIa could induce the expression of growth regulators that act downstream to induce cellular processes. To investigate this possibility, in the present study, we have examined changes in the transcriptional program in human fibroblasts in response to exposure to VIIa using a cDNA microarray that contain more than 8,000 individual human genes. We chose fibroblasts since these cells normally encounter serum, which contain growth factors and activated clotting factors in the context of vascular injury due to physical (e.g., surgery) and pathophysiological conditions. The temporal program of gene expression observed in response to serum suggests that fibroblasts are programmed to interpret the abrupt exposure to serum not as a general mitogenic stimulus but as a specific physiological signal (24). Characterization of transcriptional activation in response to serum and growth factors also suggest that fibroblasts are an active participants in a conversation among the diverse cells which collectively control inflammation, angiogenesis and wound healing (24).

cDNA microarray analysis with mRNA isolated from fibroblasts exposed to VIIa for 90 min suggested possible upregulation of *Cyr61*. Northern blot analysis confirmed the VIIa-induced expression of *Cyr61* in fibroblasts. Although not as robust as in fibroblasts, VIIa-also increases the expression of *Cyr61* in vascular smooth muscle cells (data not shown). Induction of *Cyr61* expression is dependent on the VIIa's catalytic activity since VIIai fail to induce the expression of *Cyr61*. Although factor Xa and thrombin could also induce the expression of *Cyr61* (data not shown), it is unlikely that they are involved in FVIIa-induced expression of *Cyr61*. We found no evidence for the generation of traces factor Xa and thrombin in our experimental system. Further, specific inhibitor of factor Xa and thrombin had no significant effect on the VIIa-induced expression of *Cyr61*. Thus it appears that the catalytic activity of VIIa may be required to activate a putative proteinase-activated receptor. However, we can not rule out the possibility that active-site of VIIa may be playing an important role in induction of *Cyr61* via TF cytoplasmic tail.

Cyr61 is an immediate-early gene that is transcriptionally activated by serum growth factors in fibroblasts (21). It encodes a secreted 40 kDa, cysteine-rich and heparin binding protein that associates with extracellular matrix and cell surfaces (25). *Cyr61* is a member of an emerging gene family of conserved and modular proteins characterized by the presence of an N-terminal secretory signal, followed by four modular structural domains and 38 cysteine residues that are largely conserved among members of the family (20). The protein family now consists of six distinct members, including Cyr61, connective tissue growth factor (CTGF) and an avian proto-oncoprotein, Nov (thus named as CCN family) (20). Cyr61 protein is shown to (i) promote the attachment and spreading of endothelial cells in a manner similar to that of fibronectin, (ii) enhance the effects of bFGF and PDGF on the rate of DNA synthesis of fibroblasts and vascular endothelial cells (iii) promotes cell migration in both fibroblasts and endothelial cells (26). Recent studies show that Cyr61 acts as a ligand to integrin $\alpha_v\beta_3$ (27), an adhesion receptor known to be involved in signaling that regulates a number of cellular processes including angiogenesis and tumor metastasis (28,29). Purified Cyr61 protein was shown to stimulate directed migration of human microvascular endothelial cell in culture through an $\alpha_v\beta_3$-dependent pathway and induce neovascularization in rat corneas (30). Furthermore, expression of *Cyr61* in tumor cells

promotes tumor growth and vascularization (30).

Based on the present data that show VIIa induces *Cyr61* expression in fibroblasts, it is possible that VIIa-induced Cyr61 may be responsible, acting through integrin $\alpha_v\beta_3$, for VIIa-mediated cell migration and tumor metastasis. Thus, Cyr61 could link VIIa-TF proteolytical signal to the integrin signalling pathway. The observations that VIIa catalytic activity is required for migration of smooth muscle cells (31) and tumor cells (19), and tumor metastasis (6) are consistent with the our observation that VIIa catalytic activity is required for the induction of *Cyr61*.

In addition to Cyr61, VIIa could also induce other regulators that could mediate VIIa-induced biological responses. VIIa binding to cell surface TF in pancreatic cancer cells was shown to selectively overexpress uPAR gene (19). Earlier we have shown, using differential display technique, up-regulation of transcription of poly(A)polymerase gene in fibroblasts exposed to VIIa (17). Although it would have been interesting to find out whether the cDNA microarray also show differential expression of PAP, the filter did not contain the PAP cDNA. In addition to *Cyr61*, our cDNA microarray also show differential expression of four other genes (see results), but the differential expression ratio was very close to the borderline significance. Since in preliminary experiments we could not confirm their differential expression by Northern blot analysis and also the absence of any suggestive relevant data on the ability of these gene products to mediate VIIa-induced biological responses, we did not analyze their expression further. However, since CTGF is a structurally related molecule to Cyr61 and elicit same biological responses as Cyr61, we have examined the expression of CTGF even though the relative ratio of CTGF expression in VIIa-treated sample vs the control sample in the cDNA microarray is 1.8 (2 is a conservative estimate to be a real magnitude in the assay). The data revcaled that VIIa also induced the expression of *CTGF* and the kinetics of VIIa-induced expression of *CTGF* was similar to that of *Cyr61* (Fig. 7).

Although CTGF behaves very similar to Cyr61, subtle differences exists between them (see (20)). For example, (a) CTGF has shown to be mitogenic in itself whereas Cyr61 has no intrinsic mitogenic activity but augments growth factor-induced DNA synthesis (b) Cyr61 stimulates chemotaxis whereas CTGF stimulates

both chemotaxis and chemokinesis (c) although both Cyr61 and CTGF are ECM-associated signaling molecules, CTGF is shown to secrete into culture medium (32). Thus, it is possible that VIIa regulates cellular functions locally via Cyr61 whereas acts at a distance from its site through the secretion of CTGF.

Finally, we are fully aware that a cDNA microarray performed with a single set of samples will not provide a global view of transcriptional events that underlies the VIIa-induced responses. This requires analyzing transcription changes in cells exposed to varying time periods. Nonetheless, our observations that VIIa-induces the expression *Cyr61* and *CTGF* and the fact that these molecules are shown to promote a diverse biological functions, including cell proliferation, cell migration, angiogenesis and tumor metastasis via integrin $\alpha_v\beta_3$, opens a possibility, not known previously, that VIIa proteolytical signalling pathway is linked to the integrin signalling pathway.

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FIGURE LEGENDS

Fig. 1. *Northern blot analysis confirming the data obtained with cDNA microarray assay.* Ten μg of total RNA (from the same RNA samples that were used to isolate poly (A) RNA to generate probes for hybridization of cDNA microarray) were subjected to Northern blot analysis and probed with ^{32}P-labeled *Cyr61* (a partial-length cDNA, obtained from Genomic Systems). Panel B. The hybridization signals are quantified with PhosphorImager (Molecular Dynamics).

Fig. 2. *Time-dependent factor VIIa-induced expression of Cyr61.* Quiescent monolayers of WI-38 cells were treated with factor VIIa (5 μg/ml) (Panel A) or PDGF-BB (10 ng/ml) (Panel C) for varying time periods. Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled *Cyr61*. Ethidium bromide staining of 28S ribosomal RNA of the corresponding blot is shown in the bottom panel as RNA loading control.

Fig.3. *Dose-dependent factor VIIa-induced expression of Cyr61.* Quiescent monolayers of WI-38 cells were treated with varying doses of factor VIIa, 0, 0.1, 0.5, 2.0 and 5.0 μg/ml for 45 min. Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled *Cyr61*. Ethidium bromide staining of 28S ribosomal RNA of the corresponding blot is shown in the bottom panel as RNA loading control.

Fig. 4. *Factor VIIa catalytic activity is required for the induced expression of Cyr61.* Quiescent monolayers of WI-38 cells were treated with a control serum-free medium or serum-free medium containing factor VIIa (5 μg/ml) or active-site inactivated factor VIIa (VIIai, 5 μg/ml) for 45 min. Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled *Cyr61*. Ethidium bromide staining of 28S ribosomal RNA of the corresponding blot is shown in the bottom panel as RNA loading control.

Fig. 5. *Factor VIIa-induced expression of Cyr61 is not abolished by specific inhibitors of factor Xa and*

thrombin. Quiescent monolayers of WI-38 cells were treated with control medium or the medium containing factor VIIa (5 μg/ml; 100 nM)) for 45 min. Cells were preincubated with 200 nM recombinant TAP (lane 3) or hirudin (lane 4) for 30 min before exposure to factor VIIa for 45 min. Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled *Cyr61*. Ethidium bromide staining of 28S ribosomal RNA of the corresponding blot is shown in the bottom panel as RNA loading control.

Fig. 6. *Effect of actinomycin-D and cycloheximide on factor VIIa-induced Cyr61 mRNA steady-state levels.* Quiescent monolayers of WI-38 cells were preincubated with a control vehicle, actinomycin D (10 μg/ml) or cycloheximide (10 μg/ml) for 30 min before the cells were exposed to factor VIIa (5 μg/ml) for 45 min. Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled *Cyr61*. Ethidium bromide staining of 28S ribosomal RNA of the corresponding blot is shown in the bottom panel as RNA loading control.

Fig. 7. Factor VIIa induces the expression of *CTGF*.

CLAIMS

1. A method of regulating the expression of at least one gene in a cell, comprising the steps of:
   a) contacting said cell with factor VII(a) or a tissue factor antagonist
   b) determining the expression of said gene in said cell.

2. The method of claim 1, wherein said cell is a human vascular cell expressing tissue factor, including fibroblasts and smooth muscle cells.

3. The method of claim 1, wherein said gene is selected from the group consisting of *Cyr61*, CTFG, dopamine D2 receptor, EST *Incyte PD 395116* or P2U nucleotide receptor.

4. The method of claim 1, wherein said tissue factor antagonist is modified factor VII(a) known as factor VIIai.

5. A method of enhancing the expression induced according to claim 1 to 4.

6. A method of inhibiting the expression induced according to claim 1 to 4.

7. A method of enhancing according to claim 5 comprising contacting the cell with factor VII(a).

8. A method of inhibiting according to claim 6 comprising contacting the cell with modified factor VII known as VIIai.

9. The method of claim 3 wherein said gene is *EST PD674714*.

10. A method of enhancing said gene *EST PD674714* by contacting the cell with modified factor VII(a) known as VIIai.

11. A method of inhibiting said gene *EST PD674714* by contacting the cell with factor VII(a).

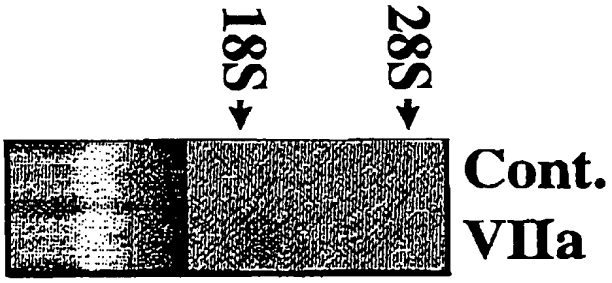

Figure 1

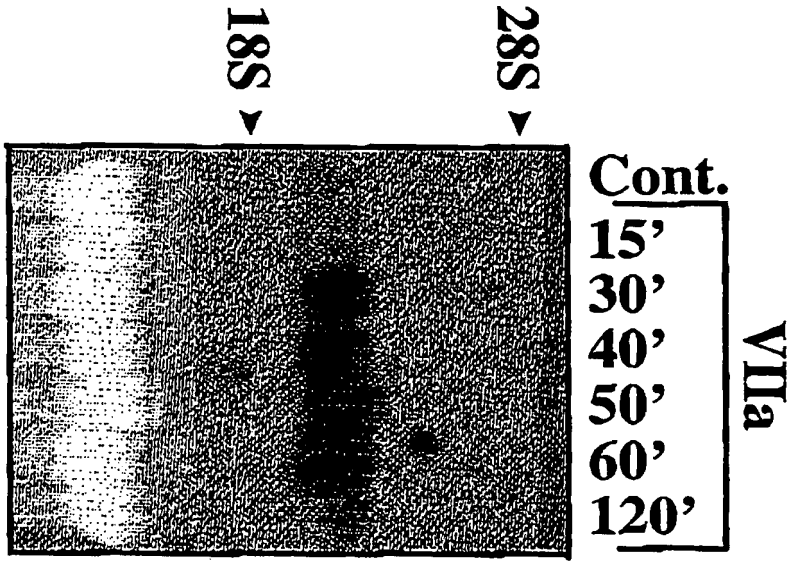

Figure 2

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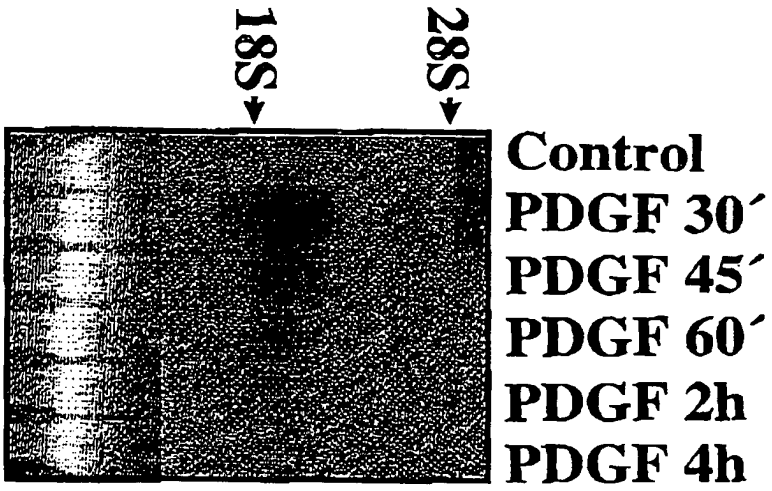

Figure 2B

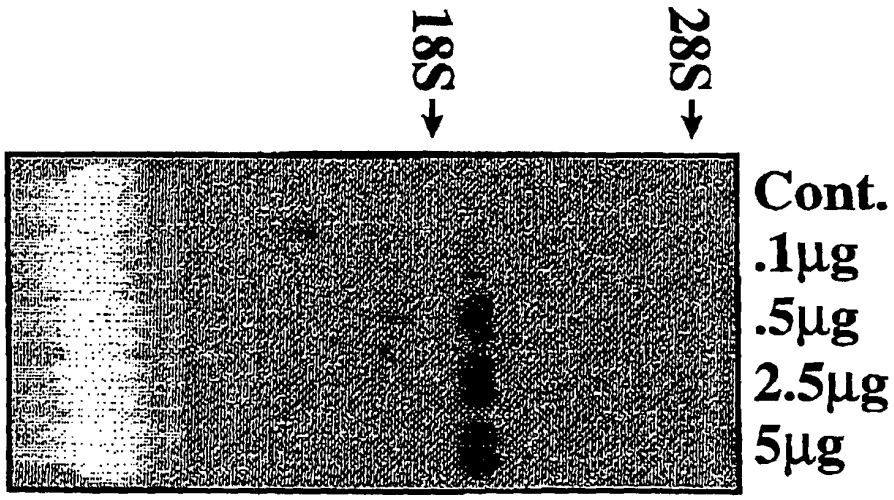

Figure 3

Figure 4

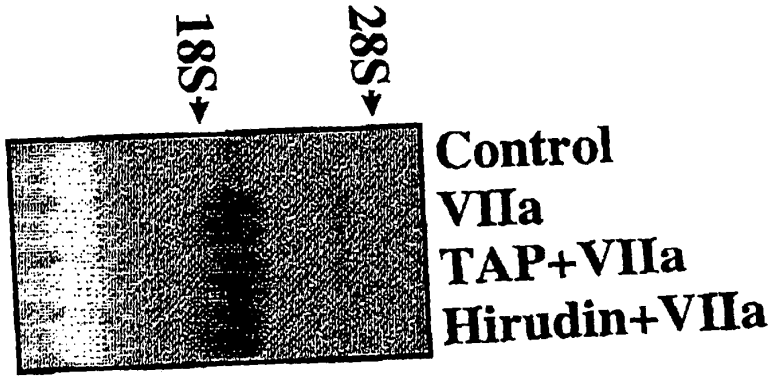

Figure 5

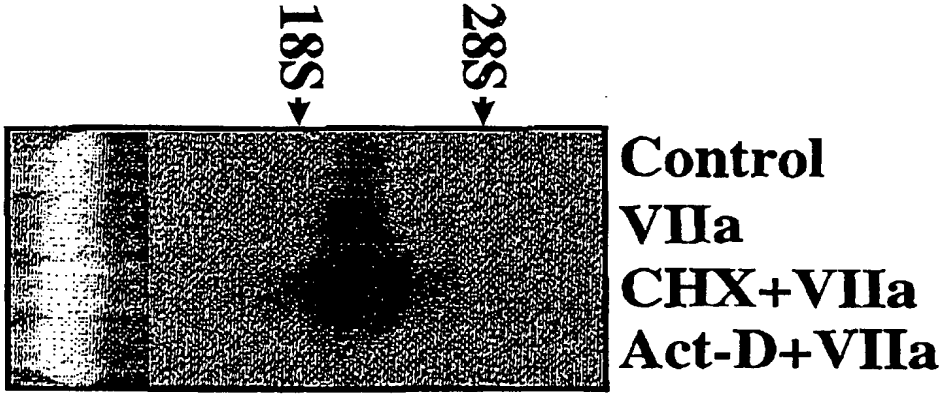

Figure 6

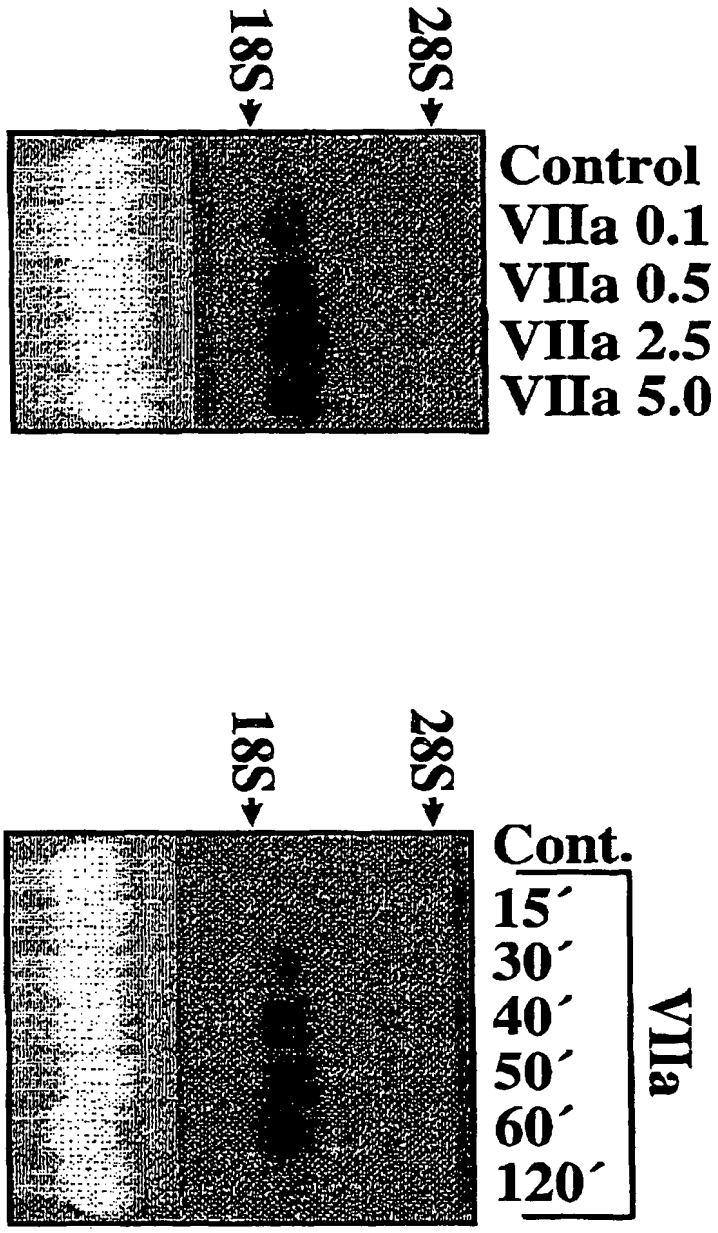

Figure 7